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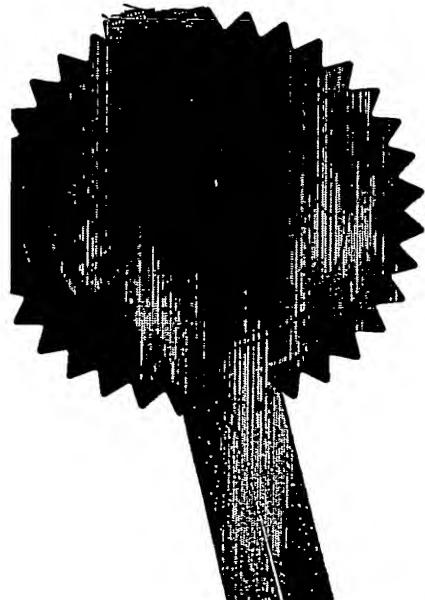
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NO. 240

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P102131GB

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0308088.4

- 9 APR 2003

3. Full name, address and postcode of the or of each applicant (underline all surnames)Asterion Limited
Firth Court
Western Bank
Sheffield
S10 2TN
GB

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

GB

82 44857001

4. Title of the invention

Polypeptide

5. Name of your agent (if you have one)

Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

31 St Saviourgate
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Claim(s) 3

Abstract 1

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Rob Docherty

01904 732120

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DUPLICATE

Polypeptide

The invention relates to chimeric recombinant polypeptides, preferably therapeutic polypeptides, which are engineered to include a signal sequence for the attachment of 5 glycosylphosphatidylinositol; cells expressing said polypeptides and methods to manufacture said polypeptides.

GPI-anchors are post-translational modifications to proteins that add glycosyl-phosphatidylinositol which enable these proteins to anchor to the extracellular side of 10 cell membranes. Typically, extracellular proteins which have a GPI anchor do not have transmembrane or cytoplasmic domains. GPI anchor proteins occur in all eukaryotes and form a diverse variety of proteins that includes by example and not by way of limitation, membrane associated enzymes, adhesion molecules and proteins which coat the outer surface of protozoan parasites such as *Trypanosoma brucei* spp. 15 The human kidney includes a number of examples of GPI-anchored proteins i.e. uromodulin, carbonic anhydrase type IV, alkaline phosphatase, Thy-1, BP-3, amino peptidase P, and dipeptidylpeptidase.

All GPI-anchor proteins are initially synthesized with a transmembrane anchor 20 which, after translocation across the endoplasmic reticulum, is cleaved and covalently linked to a preformed GPI anchor by a specific transamidase enzyme. The modification of proteins by the addition of a GPI-anchor confers important properties on the protein since the addition of the lipid moiety allows the protein to be inserted 25 into cell membranes thereby anchoring the protein thus increasing its effective local concentration.

There are some general requirements for creating a synthetic GPI anchor sequence. These are a hydrophobic region at the C-terminus of the molecule (10-20 amino acids) not followed by a cluster of basic residues, a "spacer domain" of 7-10 residues preceding the hydrophobic region and small amino acids after the spacer region, 30 where cleavage of the precursor and attachment of the anchor occurs. The GPI anchor is preassembled and added to nascent protein in the endoplasmic reticulum.

Concomitant with this step, the initial C-terminal peptide is removed so that the GPI anchor is covalently attached to a new C-terminal amino acid on the protein.

The large scale production of recombinant proteins requires a high standard of quality control since many of these proteins are used as pharmaceuticals, for example: growth hormone; leptin; erythropoietin; prolactin; TNF, interleukins (IL), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11; the p35 subunit of IL-12, IL-13, IL-15; granulocyte colony stimulating factor (G-CSF); granulocyte macrophage colony stimulating factor (GM-CSF); ciliary neurotrophic factor (CNTF); cardiotrophin-1 (CT-1); leukemia inhibitory factor (LIF); oncostatin M (OSM); interferon, IFN α , IFN γ , and extracellular receptor domains from any cell surface receptor. Moreover, the development of vaccines, particularly subunit vaccines, (vaccines based on a defined antigen, for example gp120 of HIV), requires the production of large amounts of pure protein free from contaminating antigens which may provoke anaphylaxis.

15 The production of recombinant protein in cell expression systems is based either on prokaryotic cell expression or eukaryotic cell expression. The latter is preferred when post-translation modifications to the protein are required. Eukaryotic systems include the use of mammalian cells, e.g. Chinese Hamster Ovary cells; insect cells e.g. *Spodoptera* spp; or yeast e.g. *Saccharomyces* spp, *Pichia* spp.

We disclose recombinant proteins which are adapted by the addition of a signal sequence for the attachment of glycosylphosphatidylinositol. Transfected cells expressing said adapted protein retain biological activity and advantageously can be purified with relative ease due to their location in the cell membrane (and/or shedding into the culture media facilitating continuous culture methods) of cells transfected with nucleic acid molecules, typically vectors, which express these proteins.

30 According to an aspect of the invention there is provided a chimeric polypeptide wherein said polypeptide is engineered to include a domain comprising at least one

heterologous signal sequence which sequence directs the attachment of at least one glycosylphosphatidylinositol molecule.

In our co-pending application, PCT/GB02/04665, currently unpublished, we disclose
5 antagonistic chimeric polypeptides which comprise the fusion of a ligand binding domain of a cytokine receptor and a domain which includes a signal sequence for the attachment of glycosylphosphatidylinositol. The content of PCT/GB02/04665 is hereby disclaimed with respect to the present application.

10 In a further preferred embodiment of the invention said domain comprises the amino acid sequence:

PSPTPTETAT PSPTPKPTST PEETEAPSSA TTLISPLSLI VIFISFVLLI

15 In an alternative preferred embodiment of the invention said domain comprises the amino acid sequence:

LVPRGSIEGR GTSITAYNSE GESAEFFFLL ILLLLLVLV.

In a further alternative preferred embodiment of the invention said domain comprises the amino acid sequence:

20 TSITAYKSE GESAEFFFLL ILLLLLVLV.

In a preferred embodiment of the invention said polypeptide includes at least one glycosylphosphatidylinositol molecule.

25 In a further preferred embodiment of the invention said polypeptide is a therapeutic polypeptide.

Typically a therapeutic polypeptide is a polypeptide with agonistic or antagonistic activity.

30 For example, and not by way of limitation, tumor suppressor polypeptides (e.g. p53 polypeptide, the APC polypeptide, the DPC-4 polypeptide, the BRCA-1 polypeptide,

the BRCA-2 polypeptide, the WT-1 polypeptide, the retinoblastoma polypeptide (Lee, et al. (1987) Nature 329:642), the MMAC-1 polypeptide, the adenomatous polyposis coli protein (United States Patent 5,783,666), the deleted in colon carcinoma (DCC) polypeptide, the MMSC-2 polypeptide, the NF-1 polypeptide, 5 nasopharyngeal carcinoma tumour suppressor polypeptide (Cheng, et al. 1998. Proc. Nat. Acad. Sci. 95:3042-3047), the MTS1 polypeptide, the CDK4 polypeptide, the NF-1 polypeptide, the NF2 polypeptide, and the VHL polypeptide.

“Antigenic polypeptides” (e.g. tumour rejection antigens the MAGE, BAGE, GAGE 10 and DAGE families of tumour rejection antigens, see Schulz et al Proc Natl Acad Sci USA, 1991, 88, pp991-993). Antigenic polypeptides also includes polypeptide antigens used in the preparation of vaccines which provide protection against infectious agents. For example, viruses such as Human Immunodeficiency Virus (HIV1 & 2); Human T Cell Leukaemia Virus (HTLV 1 & 2); Ebola virus; Human 15 Papilloma Virus (e.g. HPV-2, HPV-5, HPV-8 HPV-16, HPV-18, HPV-31, HPV-33, HPV-52, HPV-54 and HPV-56); papovavirus; rhinovirus; poliovirus; herpesvirus; adenovirus; Epstein Barr virus; influenza virus, hepatitis B and C viruses. Antigens derived from pathogenic bacteria such as *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Enterococcus faecalis*; *Mycobacterium tuberculosis*; *Streptococcus group B*; *Streptococcus pneumoniae*; *Helicobacter pylori*; *Neisseria gonorrhoea*; *Streptococcus group A*; *Borrelia burgdorferi*; *Coccidioides immitis*; *Histoplasma capsulatum*; *Neisseria meningitidis*; *Shigella flexneri*; *Escherichia coli*; *Haemophilus influenzae*. Antigens derived from parasites such as *Trypanosoma spp*, *Plasmodia spp*, *Schiztosoma spp*; and pathogenic fungi such as *Candida spp*.

25

Therapeutic polypeptides which are “cytotoxic polypeptides” (e.g. *pseudomonas* exotoxin, ricin toxin, diphtheria toxin).

Therapeutic polypeptides which are “cytostatic polypeptides” (e.g. p21, the 30 retinoblastoma polypeptide, the E2F-Rb polypeptide, cyclin dependent kinase inhibitors such as P16, p15, p18 and p19, the growth arrest specific homeobox

(GAX) polypeptide as described in Branellec, *et al*, see WO97/16459 and WO96/30385.

Therapeutic polypeptides which are "pro-drug" activating polypeptides (e.g. cytosine deaminase).

Therapeutic polypeptides which are "apoptosis inducing" polypeptides (e.g. p53, adenovirus E3-11.6K(10.5K), the adenovirus E4orf4 polypeptide, p53 pathway polypeptides, and caspases.

Therapeutic polypeptides which are "pharmaceutical polypeptides" (cytokines e.g. growth hormone; leptin; erythropoietin; prolactin; TNF, interleukins (IL), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11; the p35 subunit of IL-12, IL-13, IL-15; granulocyte colony stimulating factor (G-CSF); granulocyte macrophage colony stimulating factor (GM-CSF); ciliary neurotrophic factor (CNTF); cardiotrophin-1 (CT-1); leukemia inhibitory factor (LIF); oncostatin M (OSM); interferon, IFN α and IFN γ , and antagonists based on extracellular domain receptor for the above cytokines or fusions of the above cytokines with their cognate extracellular domain receptor.

Therapeutic polypeptides which are "anti-angiogenic" polypeptides (e.g. angiostatin, inhibitors of vascular endothelial growth factor (VEGF) such as Tie 2 (as described in PNAS(USA)(1998) 95:8795-8800), endostatin.

Also included within the scope of therapeutic polypeptides are therapeutic antibodies. Preferably said antibodies are monoclonal antibodies or at least the active binding fragments thereof. Monoclonal antibodies maybe humanised or chimeric antibodies. The chimeric antibodies may be fusions with extracellular domain receptor.

A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody.

A humanised antibody is produced by recombinant methods to combine the complementarity determining regions (CDRs) of an antibody with both the constant

(C) regions and the framework regions from the variable (V) regions of a human antibody.

5 In a further preferred embodiment of the invention there is provided a polypeptide according-to-the-invention which has been modified by addition, deletion or substitution of at least one amino acid residue to provide a sequence variant of the polypeptide according to the invention.

10 Typically, variants include chimeras specifically modified to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of the chimera by eliminating proteolysis by proteases in an expression system.

15 The skilled person will also realize that conservative amino acid substitutions may be made in the chimeric polypeptides to provide functionally equivalent variants of the foregoing polypeptides, (i.e. the variants retain the functional capabilities of the chimeras). As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not deleteriously alter the relative charge, hydrophobicity or size characteristics of the protein in which the amino acid substitution is made.

20

25 Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups:

30 (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

(Q,N can be included, in some instances where size and polarity are conserved, but chelation is unimportant)

Conservative amino-acid substitutions in the amino acid sequence of chimeric polypeptides 5 to produce functionally equivalent variants of these polypeptides typically are made by alteration of a nucleic acid encoding the chimera. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985.

10

Alternatively, or preferably, said modification includes the use of modified amino acids in the production of recombinant or synthetic forms of chimeric polypeptides according to the invention. It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N⁶-acetyllysine, N⁶-methyllysine, N⁶,N⁶-dimethyllysine, N⁶,N⁶,N⁶-trimethyllysine, cyclohexylalanine, D-amino acids, ornithine.

Modifications which alter the biological activity of a polypeptide according to the invention are also within the scope of the invention, for example, a modification 20 which converts an agonist to an antagonist, sometimes referred to as a dominant negative mutation, or produces a super agonist. In our co-pending application, PCT/GB02/005523, currently unpublished, we disclose a variant growth hormone polypeptide which acts as an antagonist. The polypeptide disclosed in PCT/GB02/005523, which is incorporated by reference, is a chimeric polypeptide 25 comprising at least one modified binding domain of growth hormone (GH) and a growth hormone binding domain of a growth hormone receptor (GHR).

Modified GH's are disclosed in US 5, 849, 535 which is incorporated by reference. The modification to GH is at both site 1 and site 2 binding sites. The modifications to site 1 produce a GH molecule which has a higher affinity for GHR compared to 30 wild-type GH. These modified GH molecules act as agonists. There is also

disclosure of site 2 modifications which result in the creation of GH antagonists. Further examples of modifications to GH which alter the binding affinity of GH for site 1 are disclosed in US 5,854,026; US 6,004,931; US 6,022,711; US 6,057,292; and US 6136563 each of which are incorporated by reference. Modifications to site 2 are also disclosed, in particular amino acid residue G120 in GH which when modified to either arginine, lysine, tryptophan, tyrosine, phenylalanine, or glutamic acid creates a GH molecule with antagonistic properties. PCT/GB02/005523 discloses chimeric polypeptides which comprise modified growth hormone fused to the extracellular binding domain of growth hormone receptor. The chimera acts as an antagonist and has the property of reduced systemic clearance when administered to a patient in need of GH antagonist therapy.

The current application modifies those GH variants disclosed in US 5,849,535, US 5,854,026; US 6,004,931; US 6,022,711; US 6,057,292; and US 6136563 by the inclusion of a domain which comprises a heterologous signal sequence which directs the attachment of glycosylphosphatidylinositol. The current application also modifies the chimeric GH antagonist disclosed in PCT/GB02/005523 by the inclusion of said signal sequence for the attachment of glycosylphosphatidylinositol.

20 In a yet further preferred embodiment of the invention there is provided a polypeptide wherein said polypeptide comprises at least two polypeptides according to the invention which two polypeptides are linked via a linking molecule. Preferably said linking molecule is a flexible linker.

25 Preferably the linker comprises at least one copy of the peptide:

Gly Gly Gly Gly Ser (hereinafter referred to as "Gly4Ser").

In a further preferred embodiment of the invention said linker comprises at least 2, 3,
30 4 or 5 copies of said Gly4Ser linker.

In a yet further preferred embodiment of the invention said linker further comprises a protease sensitive site. Preferably said cleavage site is sensitive to a serum protease

5 Preferably said cleavage site comprises the amino acid sequence: LVPRGS, or variant thereof.

In a further preferred embodiment of the invention said cleavage site comprises at least one copy of the amino acid sequence: SGGGG, or functional variant thereof. Preferably, said cleavage site comprises the amino acid sequence PGISGGGGGG.

10 More preferably still said cleavage site comprises the amino acid sequence: LVPRGS PGISGGGGGG, or variant thereof.

15 Alternatively, said cleavage site comprises at least two copies of the amino acid sequence SGGGG, or functional variant thereof, which flank said cleavage site.

In a further preferred embodiment of the invention said cleavage site is sensitive to the serum protease thrombin.

20 In a yet further preferred embodiment of the invention there is provided an oligomeric polypeptide molecule comprising a plurality of polypeptides according to the invention.

In a preferred embodiment said protein comprises at least 3, 4, 5, 6, 7, 8, 9, or 10 polypeptides according to the invention.

25 According to a further aspect of the invention there is provided a nucleic acid molecule comprising a nucleic acid sequence which encodes a chimeric polypeptide according to the invention.

According to a yet further aspect of the invention there is provided a vector comprising a nucleic acid molecule according to the invention.

In a preferred embodiment of the invention said vector is an expression vector adapted for eukaryotic gene expression.

Typically said adaptation includes, the provision of transcription control sequences (promoter/enhancer sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive.

Promoter is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only, and not by way of limitation. Enhancer elements are *cis* acting nucleic acid sequences often found 5' to the transcription initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences and are therefore position independent). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to *trans* acting transcription factors (polypeptides) which have been shown to bind specifically to enhancer elements. The binding/activity of transcription factors (please see Eukaryotic Transcription Factors, by David S Latchman, Academic Press Ltd, San Diego) is responsive to a number of environmental cues which include, by example and not by way of limitation, intermediary metabolites (e.g. glucose, lipids), environmental effectors (e.g. heat).

20

Promoter elements also include so called TATA box and RNA polymerase initiation selection (RIS) sequences which function to select a site of transcription initiation. These sequences also bind polypeptides which function, *inter alia*, to facilitate transcription initiation selection by RNA polymerase.

25

Adaptations also include the provision of selectable markers and autonomous replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell. Vectors which are maintained autonomously are referred to as episomal vectors.

30

Adaptations which facilitate the expression of vector encoded genes include the provision of transcription termination/polyadenylation sequences. This also includes the provision of internal ribosome entry sites (IRES) which function to maximise expression of vector encoded genes arranged in bicistronic or multi-cistronic expression cassettes.

These adaptations are well known in the art. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

It will be apparent to one skilled in the art that the vectors according to the invention could be gene therapy vectors. Gene therapy vectors are typically virus based. A number of viruses are commonly used as vectors for the delivery of exogenous genes. Commonly employed vectors include recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridae, parvoviridae, picornaviridae, herpesviridae, poxviridae, adenoviridae, or picornaviridae. Chimeric vectors may also be employed which exploit advantageous elements of each of the parent vector properties (See e.g., Feng, et al.(1997) Nature Biotechnology 15:866-870). Such viral vectors may be wild-type or may be modified by recombinant DNA techniques to be replication deficient, conditionally replicating or replication competent.

Preferred vectors are derived from the adenoviral, adeno-associated viral and retroviral genomes. In the most preferred practice of the invention, the vectors are derived from the human adenovirus genome. Particularly preferred vectors are derived from the human adenovirus serotypes 2 or 5. The replicative capacity of such vectors may be attenuated (to the point of being considered "replication

deficient") by modifications or deletions in the E1a and/or E1b coding regions. Other modifications to the viral genome to achieve particular expression characteristics or permit repeat administration or lower immune response are preferred.

5

Alternatively, the viral vectors may be conditionally replicating or replication competent. Conditionally replicating viral vectors are used to achieve selective expression in particular cell types while avoiding untoward broad spectrum infection. Examples of conditionally replicating vectors are described in Pennisi, E. (1996)

10 Science 274:342-343; Russell, and S.J. (1994) Eur. J. of Cancer 30A(8): 1165-1171. Additional examples of selectively replicating vectors include those vectors wherein a gene essential for replication of the virus is under control of a promoter which is active only in a particular cell type or cell state such that in the absence of expression of such gene, the virus will not replicate. Examples of such vectors are described in
15 Henderson, et al., United States Patent No. 5,698,443 issued December 16, 1997 and Henderson, et al., United States Patent No. 5,871,726 issued February 16, 1999 the entire teachings of which are herein incorporated by reference.

20 Additionally, the viral genome may be modified to include inducible promoters which achieve replication or expression only under certain conditions. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230: 426-430; Iida, et al. (1996) J. Virol. 70(9): 6054-6059; Hwang, et al.(1997) J. Virol 71(9): 7128-7131; Lee, et al. (1997) Mol. Cell. Biol. 17(9): 5097-5105; and Dreher, et al.(1997) J. Biol. Chem
25 272(46): 29364-29371.

Vectors may also be non-viral and are available from a number of commercial sources readily available to a person skilled in the art. For example, the vectors may be plasmids which can be episomal or integrating.

According to a further aspect of the invention there is provided a cell transfected with the vector or nucleic acid molecule according to the invention.

5 In a preferred embodiment of the invention said cell is a eukaryotic cell.

Preferably said eukaryotic cell is selected from the group consisting of: a fungal cell eg *Saccharomyces cerevisiae*, *Pichia spp*; slime mold (eg *Dictyostelium spp*); insect cell (eg *Spodoptera frugiperda*); a plant cell; or a mammalian cell (e.g. CHO cell).

10

Methods to transfect cells, in particular eukaryotic cells, are well known in the art.

Transfection methods to introduce DNA into cells typically involve the use of chemical reagents, cationic lipids or physical methods. Chemical methods which facilitate the uptake of DNA by cells include the use of DEAE -Dextran (Valeri and Pagano (1965) Science 175: p434) . DEAE-dextran is a negatively charged cation which associates and introduces the DNA into cells but which can result in loss of cell viability. Calcium phosphate is also a commonly used chemical agent which when co-precipitated with DNA introduces the DNA into cells (Graham et al 15 Virology (1973) 52: p456).

The use of cationic lipids (e.g. liposomes (Felgner (1987) Proc.Natl.Acad.Sci USA, 84:p7413)) has become a common method since it does not have the degree of toxicity shown by the above described chemical methods. The cationic head of the 25 lipid associates with the negatively charged nucleic acid backbone of the DNA to be introduced. The lipid/DNA complex associates with the cell membrane and fuses with the cell to introduce the associated DNA into the cell. Liposome mediated DNA transfer has several advantages over existing methods. For example, cells which are recalcitrant to traditional chemical methods are more easily transfected using 30 liposome mediated transfer.

More recently still, physical methods to introduce DNA have become effective means to reproducibly transfect cells. Direct microinjection is one such method which can deliver DNA directly to the nucleus of a cell (Capecchi (1980) Cell, 22:p479). This allows the analysis of single-cell transfecteds. So called "biostatic" methods 5 physically shoot DNA into cells and/or organelles using a particle gun (Neumann (1982) EMBO J, 1: p841). Electroporation is arguably the most popular method to transfect DNA. The method involves the use of a high voltage electrical charge to momentarily permeabilise cell membranes making them permeable to macromolecular complexes.

10

More recently still, a method termed immunoporation has become a recognised technique for the introduction of nucleic acid into cells, (see Bildirici *et al* Nature (2000) 405, 298). The technique involves the use of beads coated with an antibody to a specific receptor. The transfection mixture includes nucleic acid, typically vector 15 DNA, antibody coated beads and cells expressing a specific cell surface receptor. The coated beads bind the cell surface receptor and when a shear force is applied to the cells the beads are stripped from the cell surface. During bead removal a transient hole is created through which nucleic acid and/or other biological molecules can enter. Transfection efficiency of between 40-50% is achievable depending on the 20 nucleic acid used.

In a further aspect of the invention there is provided a method to prepare a polypeptide according to the invention comprising;

25 (i) growing a cell transfected with a vector or nucleic acid according to the invention in conditions conducive to the manufacture of said polypeptide; and
(ii) purifying said polypeptide from said cell, or its growth environment.

It will be apparent that polypeptides according to the invention can be purified in a 30 number of ways from cells expressing nucleic acids and/or vectors according to the invention. For example, cells maybe isolated from cell growth media followed by

proteolytic cleavage of said polypeptide from the cell membrane. Alternatively, polypeptides can be secreted or cleaved in cell culture to release the polypeptide into the surrounding cell growth media. The polypeptides are then subsequently isolated from cell growth media and purified by conventional techniques (e.g. affinity chromatography, ultra centrifugation). Polypeptides can be further processed to remove the glycosylphosphatidylinositol anchor.

According to a yet further aspect of the invention there is provided a cell wherein said cell presents, at least at its cell surface, a polypeptide according to the invention.

10

It will be apparent to one skilled in the art that cells could be incubated with chimeric polypeptides as herein disclosed which would insert via the glycosylphosphatidylinositol anchor and become localised in the cell membrane. These cells could then act as delivery vehicles for said polypeptides when administered to patients in need of treatment. For example, polypeptides according to the invention could be incubated with red blood cells taken from a patient, which is subsequently re-administered to the patient.

Also polypeptides according to the invention may insert into a cell membrane after administration (i.e. if injected, into a joint or systemically the GPI containing polypeptide may insert into cell membranes. GPI containing cytokine antagonists may be injected into a coronary artery to block a local cytokine effect. A common problem now is restenosis of coronary arteries after they have been dilated blocking the inflammatory response by local injection of a cytokine antagonist is desirable. The insertion could either be through administering peptide itself or getting local expression by applying a DNA encoding for the peptide-GPI.

In a yet further aspect of the invention there is provided a polypeptide, a nucleic acid molecule, a vector or a cell according to the invention for use as a pharmaceutical.

Preferably said polypeptide, nucleic acid molecule, vector or cell is used in a pharmaceutical composition.

When administered the pharmaceuticals/compositions of the present invention is administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents.

5

The pharmaceuticals/compositions of the invention can be administered by any conventional route, including injection. The administration and application may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, intra-articular, subcutaneous, topical (eyes), dermal (e.g a cream lipid soluble insert into skin or mucus membrane) or transdermal.

10

Pharmaceuticals/compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a pharmaceuticals/compositions that alone, or together with further doses or synergistic drugs, produces the desired response.

15 This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods or can be monitored according to diagnostic methods.

20 The doses of the pharmaceuticals/compositions administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject (i.e. age, sex). When administered, the pharmaceuticals/compositions of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions.

25 Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention.

30 Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric,

hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

5

The pharmaceuticals/compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceuticals/compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

20

The pharmaceuticals/compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

25

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or 5 non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using 10 suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are 15 conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack 20 Publishing Co., Easton, PA.

Advantageously, pharmaceutical preparations comprising polypeptides according to the invention are able to form micelles due to the presence of glycosylphosphatidylinositol anchors. The formation of micelles, either *in vitro* prior to administration, or *in vivo*, after administration, enables the formation of large 25 complexes which have reduced clearance rates. This allows the use of lower effective doses of polypeptide to be administered thereby reducing harmful side-effects.

An analogous effect is shown if the polypeptides according to the invention are incorporated into liposomes. Liposomes are lipid based vesicles which encapsulate a 30 selected therapeutic agent which is then introduced into a patient. The liposome is

manufactured either from pure phospholipid or a mixture of phospholipid and phosphoglyceride. Typically liposomes can be manufactured with diameters of less than 200nm, this enables them to be intravenously injected and able to pass through the pulmonary capillary bed. Furthermore the biochemical nature of liposomes confers permeability across blood vessel membranes to gain access to selected tissues. Liposomes do have a relatively short half-life. So called STEALTH^R liposomes have been developed which comprise liposomes coated in polyethylene glycol (PEG). The PEG treated liposomes have a significantly increased half-life when administered intravenously to a patient. In addition, STEALTH^R liposomes show reduced uptake in the reticuloendothelial system and enhanced accumulation in selected tissues. In addition, so called immuno-liposomes have been developed which combine lipid based vesicles with an antibody or antibodies, to increase the specificity of the delivery of the agent to a selected cell/tissue.

The use of liposomes as delivery means is described in US 5580575 and US 5542935.

According to a yet further aspect of the invention there is provided a method of treatment of an animal, preferably a human, comprising administering an effective amount of a nucleic acid and/or vector and/or polypeptide and /or cell according to the invention.

An embodiment of the invention will now be described by example only and with reference to the following figures;

Figure 1 illustrates the cloning strategy to generate GPI linked proteins.

The gene of interest is positioned between a *Nde*I ("sticky" cutter) and *Eco*RV/*Xma*I (blunt cutter) restriction sites by PCR. The PCR product is then digested with *Nde*I and *Eco*RV/*Xma*I, the resulting product is ligated between *Nde*I and *Eco*RV sites in pCR-3/GHRss-GPI to obtain a vector from which a GPI linked protein may be expressed;

Figure 2 illustrates the nucleotide and amino acid sequence of the GH-GPI construct. Sequence from the original pCR-3/GPI vector is shown underlined, linker sequence between the promoter and the initiation codon, *ATG*, is shown in white on black and the subsequent GHR signal sequence is in *similar colours but italicised*. The GH sequence is shown in CAPITALS and the link between the GH protein and the GPI anchor shown in black on grey, the GPI anchor signal sequence is shown *italicised and underlined*. All the relevant restrictions sites are in bold and include *BamHI* (ggatcc), *NdeI* (catatg) and *EcoRV* (gatatac);

5

10 Figure 3 illustrates the nucleotide and amino acid sequence of the 1B1-GPI construct (1B1 is GH linked to GHR). Sequence from the original pCR-3/GPI vector is shown underlined, linker sequence between the promoter and the initiation codon, *ATG*, is shown in white on black and the subsequent GHR signal sequence is shown in *similar colours but italicised*. The 1B1 sequence is shown in CAPITALS and the link between the 1B1 protein and the GPI anchor shown in black on grey, the GPI anchor signal sequence is shown *italicised and underlined*. All the relevant restrictions sites are in bold and include *BamHI* (ggatcc), *NdeI* (catatg) and *XmaI* (cccgcc);

15

20 Figure 4 illustrates the nucleotide and amino acid sequence of the 1C1-GPI construct (1C1 is GH linked to GH as a tandem). Sequence from the original pCR-3/GPI vector is shown underlined, linker sequence between the promoter and the initiation codon, *ATG*, is shown in white on black and the subsequent GHR signal sequence is shown in *similar colours but italicised*. The 1C1 sequence is shown in CAPITALS and the link between the 1C1 protein and the GPI anchor shown in black on grey, the GPI anchor signal sequence is shown *italicised and underlined*. All the relevant restrictions sites are in bold and include *BamHI* (ggatcc), *NdeI* (catatg) and *EcoRV* (gatatac);

25

30 Figure 5 illustrates a charcoal assay for GHBP. The assay shows a comparison of the amounts of GHBP released into the medium from transient and stable transfected

CHO cells after 48 hours. In all cases the medium was concentrated 20 times using a Centricon 30 column prior to the charcoal assay being carried out; and

Figure 6 illustrates a western blot of samples from the membrane purification of
5 GHBP-GPI expressed in CHO cells. The western blot was generated by probing
with mouse anti-GHBP antibodies (2C8 + 263) and then probing with peroxidase
labelled anti-mouse IgG. The lanes on the blot contain - 1. Positive control: GHBP-
GPI (stable clone), 2. Untransfected cells (P1), 3. Untransfected cells (P2), 4.
10 Untransfected cells (S1), 5. Molecular weight standards (25, 37, 50, 75, 100, 150,
200 kDa), 6. Medium from untransfected cells, 7. GHBP-GPI (stable clone) (P1), 8.
GHBP-GPI (stable clone) (P2), 9. GHBP-GPI (stable clone) (S1) and 10. Medium
from GHBP-GPI (stable clone). The blot shows that GHBP-GPI has been purified
successfully in the membrane prep (Lane 8).

Materials and Methods

15 **Cloning Strategy**

The GHR signal sequence was first inserted into the vector, pCR-3/GPI, to enable the subsequently expressed proteins to be targeted to the cell membrane. The GHR signal sequence flanked by a *Bam*HI site and (*Nde*I-*Eco*RV) sites was obtained by PCR
20 using the primers GHRss_for1 and GHRss_rev1. This insert was ligated into pCR-
3/GPI between the *Bam*HI and *Eco*RV sites.

25 The protein of interest was then ligated in-frame between the *Nde*I and *Eco*RV restriction sites between the GHR signal sequence and the Thy-1 (GPI anchor signal sequence). PCR was used to generate suitable restriction sites at either end of the gene encoding the protein of interest, a *Bam*HI site was used upstream of the gene and a blunt-cutting restriction enzyme (*Eco*RV or *Xba*I) was used downstream of the gene being sub-cloned (Figure 1).

30 a) GH-GPI (Figure 2)

5 The primers GH2GPI_for1 and GH2GPI_rev1 (Table 1) were used in a PCR reaction to amplify the hGH gene flanked by *Nde*I and *Eco*RV sites. The resulting PCR product was digested with these restriction enzymes and then ligated into *Nde*I/*Eco*RV double-digested pCR3-GPI. This was then ligated into *E. coli*-XL1 Blue cells.

b) 1B1-GPI (Figure 3). 1B1 is GH linked through its C-terminus to the extracellular domain of the GH receptor and the linked to the GPI signal sequence. Since the 1B1 gene already contains the *Eco*RV restriction site, the insert was generated between 10 *Nde*I and *Xma*I restriction sites using the primers GH2GPI_for1 and 1B12GPI_rev1 (Table 1). The resulting PCR product was digested with these restriction enzymes and then ligated into *Nde*I/*Eco*RV double-digested pCR3-GPI. This was then ligated into *E. coli* XL1 Blue cells.

15 c) 1C1-GPI (Figure 4). 1C1 is a tandem of GH linked through the second GH C-terminus to the GPI signal sequence. The primers GH2GPI_for1 and GH2GPI_rev1 (Table 1) were used in a PCR reaction to amplify the 1C1 gene flanked by *Nde*I and *Eco*RV sites. The resulting PCR product was digested with these restriction enzymes and then ligated into *Nde*I/*Eco*RV double-digested pCR3-GPI. This was then ligated 20 into *E. coli* SURE cells.

Transient Transfection into CHO cells

CHO cells were grown to 70% confluence and then transfected with 3 μ g of vector 25 (e.g. pCR-3/GHBP-GPI) using the LT1 Reagent Kit (Corrus Scientific Ltd.). The cells were then grown overnight at 37°C.

Stable Transfection into CHO cells

30 CHO cells were grown to 70% confluence and then transfected with 8 μ g of vector (e.g. pCR-3/GHBP-GPI) using the Fugene 6 methodology (Roche). After 24 hours incubation the media on the cells was replaced with selective media (CHO cell media

with 400 μ g/ml G418), if required the cells were split (1:3) onto fresh 100mm dishes.
The dishes were incubated for a further 2-3 days.

5 The media was once again replaced with selective media, this time with 1000 μ g/ml
G418 added, and grown for a further 2 days. The cells were then split (1:10) onto
fresh dishes with selective media and incubated for a further 4 days.

10 The media was once again replaced with selective media and the dishes incubated for
a week. At this point non-transfected cells should have died off leaving only
transfected cells. The cells were then processed for FAC sorting/analysis to
determine the levels of expression.

Charcoal Assay

15 This assay is used to determine the amount of binding protein present in a liquid
medium.

20 I^{125} ligand (e.g. I^{125} GH or I^{125} Leptin) was added to a solution containing an unknown
amount of binding protein (e.g. GHBP-GPI, Obr-GPI) and was incubated overnight at
4°C on a rotating wheel. Dextran coated charcoal (Sigma, C-6197) was then added to
the tube and this incubated for 15 minutes at room temperature on a rotating wheel.
The tube was centrifuged at 13,000 rpm for 12 minutes and the supernatant removed
to a new tube. The Total Binding (TB) was then counted using a gamma-counter.

25 The Non-Specific Binding (NSB) was also measured by repeating the above
procedure but adding a large excess of "cold" ligand in addition to the I^{125} ligand.
 I^{125} ligand alone was also processed in this way, however the dextran-coated charcoal
was not added to this sample, this gave the Total Counts (TC).

30 The Percentage Specific Binding (PSB) was calculated using the following formula:-

$$PSB = (TB-NSB/TC) \times 100$$

For each sample the charcoal assay was done in triplicate and the mean PSB reported with the calculated standard error.

5 This assay was used to measure the amount of GHBP released in the medium from cells transfected (transient and stable) with pCR-3/GHBP-GPI after 48 hours, the medium was concentrated 20 times using a Centricon 30 column prior to being assayed. Medium from cells transfected with pCR-3/GPI was also processed in the same way (Figure 5).

10 Purification

a) Preparation of soluble and membranes fractions from CHO-K1 cell line stably expressing GHBP-GPI

15 CHO-K1 cells either non-transfected or expressing GHBP-GPI were grown to confluence on 100cm dishes. After serum starvation for 24-48 hrs the culture medium was removed and concentrated and desalted using a Centricon YM-10 filtration column and frozen at -80°C.

20 For preparation of membranes the cells were first washed with PBS followed by fresh PBS containing a protease inhibitor cocktail (1 μ g/ml aprotinin, antipain, pepstatin, leupeptin, 156.5 μ g/ml Benzamidine-HCl and 40 μ g/ml PMSF, this is referred to as "PBS complete"). The excess PBS was drained off and the cells scraped from the plates and lysed using a dounce homogeniser.

25 The lysate was firstly subjected to a low speed spin at 2.5k rpm for 10 minutes, 4°C. The resultant pellet contains nuclear and cellular debris (P1 fraction). The supernatant (S1 fraction) was cloudy and contained cytosolic and membrane bound fractions. The S1 fraction was collected and subjected to a high speed spin at 40k rpm for 1 hour, 4°C. The supernatant (S2 fraction, containing soluble cytosolic material) was collected and frozen at -80°C. The pellet (P2 fraction, containing insoluble membrane bound material) was washed with PBS complete and centrifuged again as before.

The P2 fraction was resuspended in PBS complete with the addition of 0.1% (v/v) Triton X-100 and frozen in aliquots -80 °C. Expression of GHBP was confirmed by Western blotting using anti GHBP antibodies (Figure 6).

5

b) Preparation of GH affinity column

GH was covalently coupled to CNBr activated sepharose by the following method: Briefly, 0.5g of CNBr activated sepharose 4 fast flow (Sigma: C-5338, lot no: 91K1548) was resuspended in 10ml ice cold 1mM HCl, the swollen matrix was then washed with 100ml of the same solution. The washed matrix was then immediately added to a 3ml solution of 0.1M NaHCO₃/0.5M NaCl, pH 8.3 (coupling buffer) containing a total of 3mg rhGH. The solution was mixed gently for 2hrs at room temperature and subsequently washed with coupling buffer. Any remaining active groups were blocked by incubation with 0.2M glycine, pH 8.0 for 2hrs at room temperature. To remove non-specifically bound protein the matrix was alternately washed with coupling buffer followed by 0.1M sodium acetate/0.5M NaCl, pH 4.5. The matrix was stored in PBS, pH7.4, containing 0.02% NaN₃ at 4 °C.

c) Purification of GHBP from CHO-K1 media

20 The GH-coupled column was equilibrated with 10x column volumes of PBS at 4 °C. Concentrated and desalting media containing GHBP was allowed to flow through the column under gravity flow. This process was repeated for up to 4 times. The column was washed with PBS and bound protein eluted with 3M KSCN again under gravity flow. The resultant eluate was desalting and concentrated using an Amicon ultrafree-25 MC centrifugal filtration unit (30,000 molecular weight cut off) and samples analysed by SDS-PAGE and western blotting techniques.

This methodology was also utilised to purify membrane bound protein, however 0.1% Triton was present in all buffers.

30

Claims

1. A chimeric polypeptide wherein said polypeptide is engineered to include a domain comprising at least one heterologous signal sequence which sequence directs the attachment of at least one glycosylphosphatidylinositol molecule.
2. A polypeptide according to Claim 1 wherein said domain comprises the amino acid sequence:
10 PSPTPTETAT PSPTPKPTST PEETEAPSSA TTLISPLSLI VIFISFVILLI.
3. A polypeptide according to Claim 1 wherein said domain comprises the amino acid sequence:
15 LVPRGSIEGR'GTSITAYNSE GESAEFFFLL ILLLLLVLV.
4. A polypeptide according to Claim 1 wherein said domain comprises the amino acid sequence:
20 TSITAYKSE GESAEFFFLL ILLLLLVLV.
5. A polypeptide according to any of Claims 1-4 wherein said polypeptide includes at least one glycosylphosphatidylinositol molecule.
6. A polypeptide according to any of Claims 1-5 wherein said polypeptide is a therapeutic polypeptide.
25
7. A polypeptide according to any of Claims 1-6 wherein said polypeptide has been modified by addition, deletion or substitution of at least one amino acid residue to provide a sequence variant of said polypeptide.
8. An oligomeric polypeptide wherein said polypeptide comprises at least two polypeptides according to any of Claims 1-7 which two polypeptides are linked via a linking molecule.
30

9. An oligomeric polypeptide according to Claim 8 wherein said linker comprises at least one copy of the peptide: Gly Gly Gly Gly Ser.

5 10. An oligomeric polypeptide according to Claim 9 wherein said linker comprises at least 2, 3, 4 or 5 copies of said linker.

11. An oligomeric polypeptide according to any of Claims 8-10 wherein said linker further comprises a protease sensitive cleavage site.

10 12. An oligomeric polypeptide according to Claim 11 wherein said cleavage site is sensitive to a serum protease.

13. An oligomeric polypeptide according to Claim 12 wherein said cleavage site 15 comprises the amino acid sequence: LVPRGS.

14. An oligomeric polypeptide according to Claim 12 wherein said cleavage site comprises the amino acid sequence PGISGGGGGGSGGGG.

20 15. An oligomeric polypeptide according to Claim 12 wherein said cleavage site comprises the amino acid sequence: LVPRGS PGISGGGGGG.

16. An oligomeric polypeptide according to Claim 12 wherein said cleavage site comprises at least two copies of the amino acid sequence SGGGG which flank said 25 cleavage site.

17. A nucleic acid molecule comprising a nucleic acid sequence which encodes a polypeptide or a chimeric polypeptide according to any of Claims 1-16.

18. A vector comprising a nucleic acid molecule according to Claim 17.

19. A vector according to Claim 18 wherein said vector is an expression vector adapted for eukaryotic gene expression.

20. A cell transfected with the vector or nucleic acid according to any of Claims
5 18-19.

21. A method to prepare a polypeptide or an oligomeric polypeptide according to any of Claims 1-16 comprising:

10 i) growing a cell transfected with a nucleic acid molecule or vector according to any of Claims 17-19 in conditions conducive to the manufacture of said polypeptide; and

ii) purifying said polypeptide from said cell, or its growth environment.

22. A cell wherein said cell presents, at least at its cell surface, a polypeptide or
15 oligomeric polypeptide according to any of Claims 1-16.

23. A polypeptide or a oligomeric polypeptide or a nucleic acid molecule or a vector or cell according to any of Claims 1-20 or 22 for use as a pharmaceutical.

20 24. A method of treatment of an animal, preferably a human, comprising administering an effective amount of a nucleic acid and/or vector and/or polypeptide and /or cell according to any of Claims 1-20 or 22.

25

30

5

Abstract

Polypeptide

The invention relates to chimeric recombinant polypeptides, preferably therapeutic polypeptides, which are engineered to include a signal sequence for the attachment of 10 glycosylphosphatidylinositol; cells expressing said polypeptides and methods to manufacture said polypeptides.

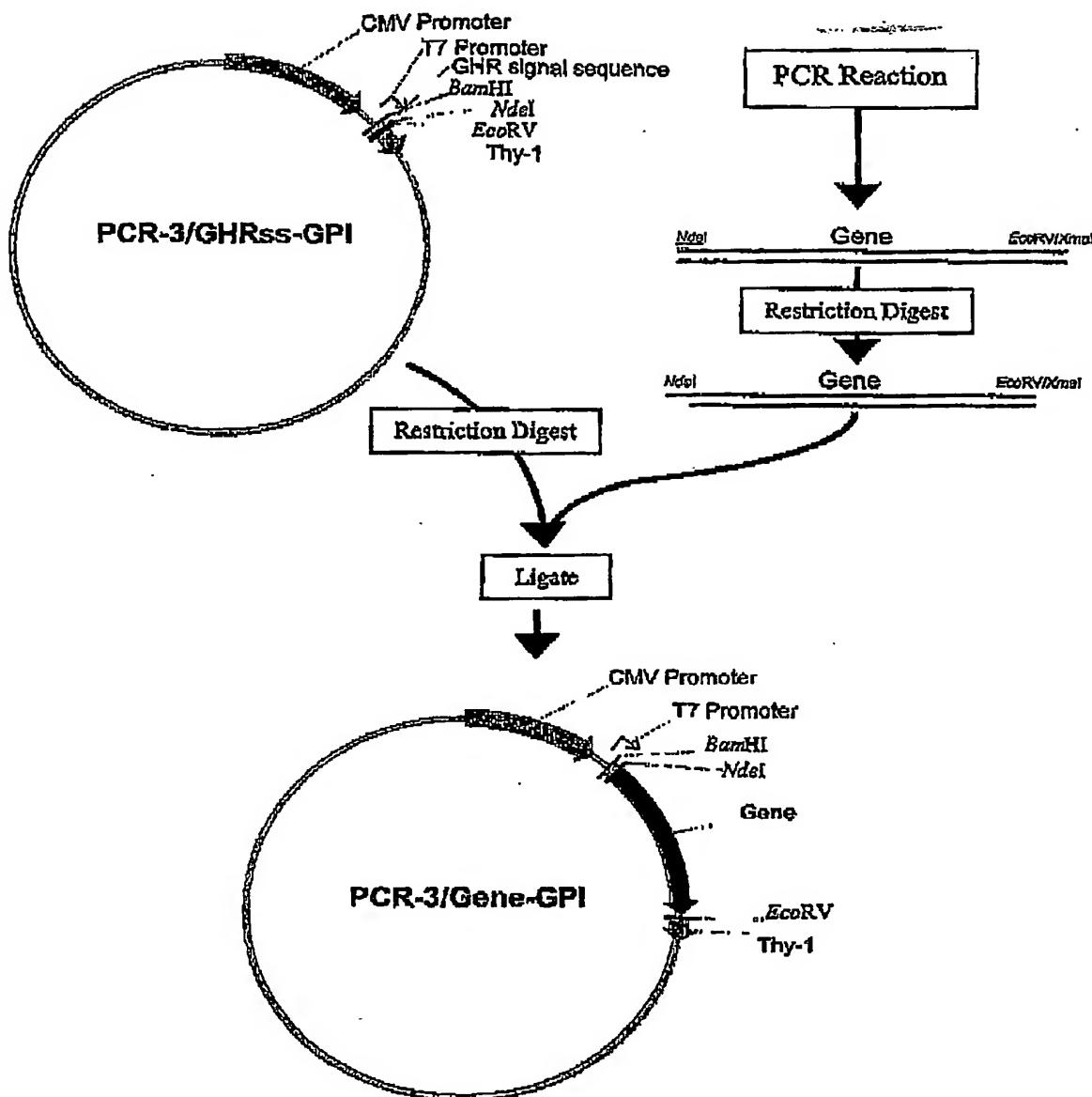


Figure 1

Nucleotide Sequence

ggatcccttagactcgagggtcctacagggtATGatatctggcagctgtgttgcac
ggcactggcaggatcaaaatgtatgtatcatatTTCCCAACCATTCCCTTATCCAGGC
TTTTGACAACGCTAGTCTCCGCCATCGTCTGCACCAGCTGGCCTTGACACCT
ACCAGGAGTTGAAGAACGCTATATCCCAAAGGAACAGAACAGTATTCAATTCTGCAGA
ACCCCCAGACCTCCCTCTGTTCTCAGAGTCTATTCCGACACCCCTCCAACAGGGAGG
AAACACAAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGT
CGTGGCTGGAGCCCCTGCAAGTCCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTACG
GCGCCTCTGACAGCAACGCTATGACCTCTAAAGGACCTAGAGGAAGGCATCAAA
CGCTGATGGGGAGGCTGGAAGATGGCAGCCCCCGGACTGGGAGATCTCAAGCAGA
CCTACAGCAAGTTGACACAAACTCACACAACGATGACGCACTACTCAAGAACTACG
GGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGTCGAGACATTCTGCGCATCG
TGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCAGCTGTGGCTTCAGCTGTGCA
AGCTGGTCAAGTGTGGCCGATAAGCCTGCTGGTTAGAACACATCCTGGATGCTGC
TGCTGCTGCTTCCCTCTCCCTCCAAGCCCTAGACTCATTCTCTGTGA

Amino Acid Sequence

MDLWQLLITLALAGSSDAHMPPPTIPLSRLFDNASLRAHRLHQIAFDTYQEFEAYIP
KEQKYSFLQNPQTSCLFSSESIPTPSNREETQQKSNLLELLRISLLLQSWLEPVQFLR
SPVANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSRTGQIFKQTYSKFDINSH
NDALLKNYGLLYCFRKDMKDVKETFLRIVQCRSVEGSCGFEDIDKLVKCGGISL
LVQNTSWMLLLLSLSLLQALDFISL*

Figure 2

Nucleotide Sequence

GgatccctctagactcgaggbcctaaggcATGgatccctcgccggatgtgtttggatct
 cggcactggcaggatcaagtgtatgtatgTTCCCAACCATTCCCTTATCCAGGC
 TTTTGACAACGCTAGTCGGCGCCCATCGTCTGCACCAGCTGGCCTTGACACCT
 ACCAGGAGTTGAAGAACGCTATATCCCAAAGGAACAGAAGTATTCAATTCTGCAGA
 ACCCCCAGACCTCCCTCTGTTCTCAGAGTCTATTCCGACACCCCTCCAACAGGGAGG
 AAACACAACAGAAATCCAACCTAGAGCTGCCATCTCCCTGCTGCTCATCCAGT
 CGTGGCTGGAGGCCGTGCAGTCCTCAGGAGTGTCTCGCCAACAGCCTGGTGTACG
 GCGCCTCTGACAGCAACGTCTATGACCTCTAAAGGACCTAGAGGAAGGCATCAAA
 CGCTGATGGGGAGGCTGGAAGATGGCAGCCCCCGACTGGCAGATCTCAAGCAGA
 CCTACAGCAAGTCGACACAAACTCACACAAAGATGACGCACTACTCAAGAAACTACG
 GGCTGCTCTACTGCTCAGGAAGGACATGGACAAGGTCGAGACATTCTGCGCATCG
 TGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCGGCGGCCGGTGGCGGAGGTA
 GTGGTGGCGGAGGTAGCGGTGGCGGAGGTCTGGTGGCGGAGGTTCCGAATTCTTT
 CTGGAAGTGAGGCCACAGCAGCTATCCTTAGCAGAGCACCCCTGGAGTCGCAAAGTG
 TTAATCCAGGCCAAAGACAAATTCTCTAAGGAGCCTAAATTCAACCAAGTGCCTGTT
 CACCTGAGCGAGAGACTTTTATGCCACTGGACAGATGAGGTTCATCATGGTACAA
 AGAACCTAGGACCCATAACAGCTGTTCTATACCAGAAGGAACACTCAAGAAATGGACTC
 AAGAATGGAAAGAATGCCCTGATTATGTTCTGCTGGGGAAAACAGCTGTTACTTTA
 ATTCACTGTTACCTCCATCTGGATACCTTATTGTATCAAGCTAACTAGCAATGGTG
 GTACAGTGGATGAAAAGTGTCTCTGTTGATGAAATAGTGCACCAAGCAGATCCACCA
 TTGCCCTCAACTGGACTTTACTGAACGTCAGTTACTGGGATTGAGATATCC
 AAGTGAGATGGGAAGCACACGCAATGCAGATATTCAAGAAAGGATGGATGGTTCTGG
 AGTATGAACTTCAATACAAAGAAGTAAATGAAACTAAATGAAAATGATGGACCCCTA
 TATTGACAACATCAGTCCAGTGTACTCATTGAAAGTGGATAAGGAATATGAAGTGC
 GTGTGAGATCCAAACAAACGAAACTCTGGAAATTATGGCGAGTTCACTGAGGTGCTCT
 ATGTAACACTTCTCAGATGAGCAATTACATGTGAAGAAGATTCTAC
 CATCGACAAAGCTGGTCAAGTGTGGGGATAAGCCTGCTGGTTCAAGAACAA
 CATCCGGATGCTGCTGCTGCTGCTTCCCTCTCCCTCCAAAGCCTAGACTCA
TTTCTCTGTGA

Amino Acid Sequence

MDIWONLELA-ACSSDHMFPTIPLSRLDNASLRAHRLHQLAFDTYQEFEEAVI
 KEQKYSFLQNPTSLCPSEIPTPSNREETOQKSNEELLRISSLILQSWLEPVQFLR
 SVFANSLVYGASDSNVYDLLKDLEEGIQTLMRLEDGSPTGQIFKQTYSKFDINSH
 NDDALLKNYGLLYCFRKDMKVETFLRIVQCRSVEGSCFGGRGGGGSGGGGGGGGG
 SGGGGSEFFSGSEATAILSRAPWSLQSVNPGLKINSSKEPKFTKRSPERETFSCH
 WTDEVHHGTIXMLGPQLFYTRRNTQEWTQEWKECPDYVSAGENSCYFNSSFTSIWIIP
 YCIKILTSNGGTVDCKFSVDEIVQPDPPIALNWTLNVSLTGIIHADIQVRWEAPRNA
 DIQKGWMVLEYELQYKEVNETWKMMDPILTTSPVYSLKVDKEYEVVRVRSKQRNSG
 NYGEFSEVLYVTLPMQSQTCEEDFYGGEEIDKLVKCGISLLVQNTSWMLLL
LSLLQALDFISL*

Figure 3

Nucleotide Sequence

GgatcccttagactcgaggccacaggtATGgatccctggcagctgtgtttgacct
 tggcactggcaggatcaagtgtatcatatgTTCCCAACCATTCCCTTATCCAGGC
 TTTTGACAACGCTAGTCTCCGGCCCCATCGTCTGCACCAGCTGGCCTTGAACACCT
 ACCAGGAGTTGAAGAACGCTATATCCC AAGGAACAGAAAGTATTCAATTCTGCAGA
 ACCCCCAGACCTCCCTCTGTTCTCAGAGTCTATTCCGACACCCCTCCAACAGGGAGG
 AAACACAAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGT
 CGTGGCTGGAGGCCGTGCAGTTCTCAGGAGTGTCTCGCCAACAGCCTGGTGTACG
 GCGCCTCTGACAGCAACGCTATGACCTCTAAAGGACCTAGAGGAAGGCATCCAAA
 CGCTGATGGGAGGCTGGAAGATGGCAGCCCCCGGACTGGCAGATCTCAAGCAGA
 CCTACAGCAAGTTGACACAAACTCACACAACGATGACGCACTACTCAAGAACTACG
 GGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGTCGAGACATTCTGCGCATCG
 TGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTCGGCGGCCGGTGGCGGAGGTA
 GTGGTGGCGGAGGTAGCGGTGGCGGAGGTTCTGGTGGCGGAGGTTCCGAATTCTTCC
 CAACCAATTCCCTTATCCAGGTTTGAACACGCTAGTCTCCGCCCCATCGTCTGC
 ACCAGCTGGCCTTGACACCTACCAAGGAGTTGAAGAACGCTATATCCAAAGGAAC
 AGAAGTATTCAATTCTGAGAACCCCCAGACCTCCCTCTGTTCTCAGAGTCTATTG
 CGACACCCCTCCAACAGGGAGGAAACACAACAGAAATCCAACCTAGAGCTGCTCCGCA
 TCTCCCTGCTGTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCTCAGGAGTGTCT
 TCGCCAACAGCCTGGTGTACGGCGCTCTCACAGCAACGCTATGACCTCTAAAGG
 ACCTAGAGGAAGGCATCAAACGCTGATGGGAGGCTGGAAGATGGCAGCCCCCGGA
 CTGGCAGATCTCAAGCAGACCTACAGCAAGTTGACACAAACTCACACAACGATG
 ACGCACTACTCAAGAACTACGGGCTGCTACTGCTTCAGGAAGGACATGGACAAGG
 TCGAGACATTCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTGGCTTC
~~ATGGACAACCTGGTCAAGTGTGGCGGCATAAGCCTGCTGGTCA~~
 AGAACACATCCCTGGATGCTGCTGCTGCTTTCCCTCTCCCTCCAAGCCCTAG
ACTTCATTCCTGTA

Amino Acid Sequence

MDLWQLLTLALAGS~~DAHM~~FPTIPLSRLFDNASLRAHRLHOLAFDTYQEFEAYIP
 KEQKYSFLQNPQTSLCFSESIPTPSNREETQQKSNLLELLRISLLLQSWLEPVQFLR
 SVFANSLVYGA~~SDS~~DNVYD~~L~~LKDLEEGIQTLMGRLEDGS~~PRT~~GQIFKQTYSKFD~~T~~N~~S~~H
 NDDALLKNYGLLYC~~FR~~KDM~~D~~KVETFLRIVQCRSVEGSCGFGRGGGGSGGGGGGGGG
 SG~~GGGG~~SEFFPTIPLSRLFDNASLRAHRLHOLAFDTYQEFEAYIPKEQKYSFLQNPQ
 TSLCFSESIPTPSNREETQQKSNLLELLRISLLLQSWLEPVQFLRSVFANSLVYGA
 DSNVYD~~L~~LKDLEEGIQTLMGRLEDGS~~PRT~~GQIFKQTYSKFD~~T~~N~~S~~HND~~D~~ALLKNYGLL
 YC~~FR~~KDM~~D~~KVETFLRIVQCRSVEGSCGF~~GGGG~~LDKLVKCGG~~I~~SLLVQNTSWML~~LL~~
LSLSLLQALDFISL*

Figure 4

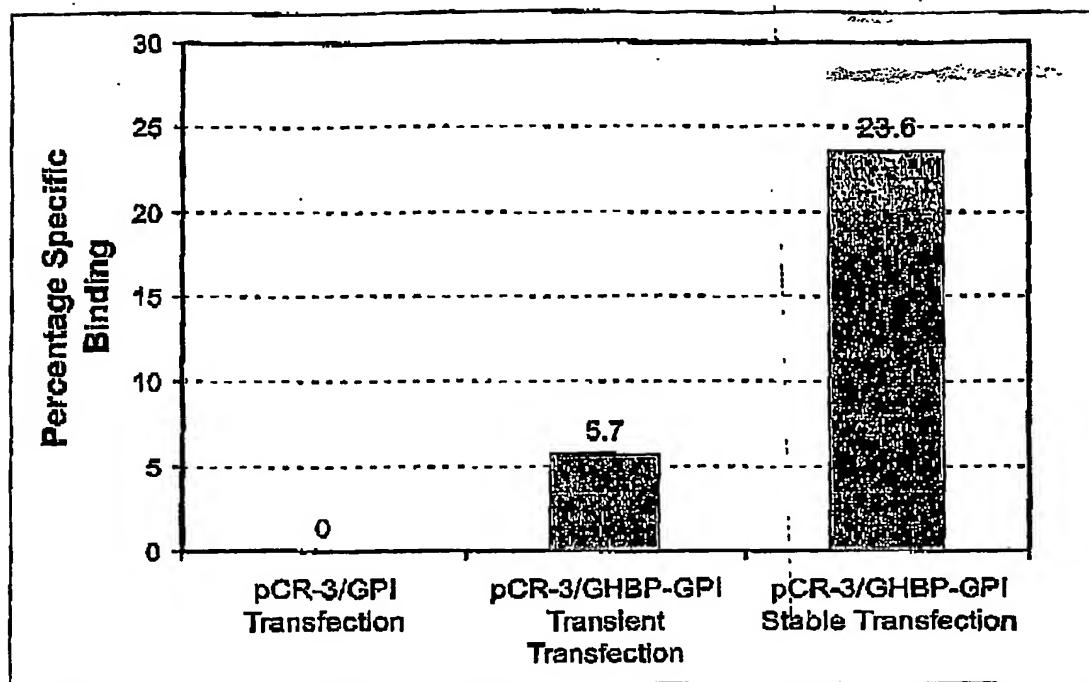


Figure 5

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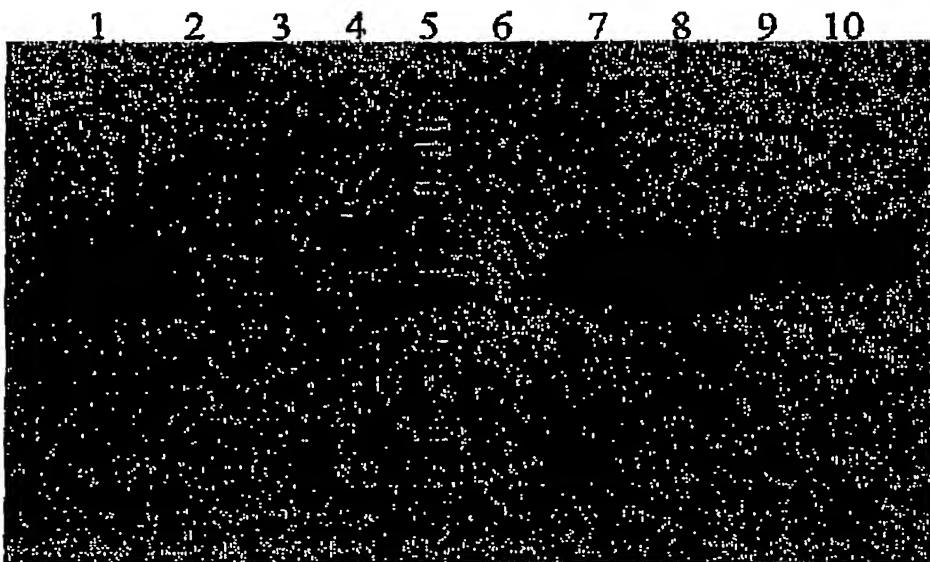
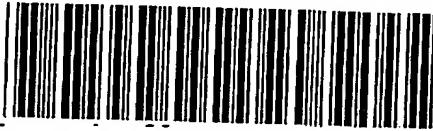


Figure 6

Primer Name	Sequence (5' → 3')
GHRss_for1	gagaggatcctctagactcgagggtctac
GHRss_rev1	gcccataatgagcatcaattgatctgag
GH2GPI_for1	gcgcataatgttcccaaccattcccttatac
GH2GPI_rev1	gcgcgatatccccccaccggccgaagccacagctgcc
1B12GPI_rev1	gcgcccgggcccctccaccggccgtagaatcttcttcacatg

Table 1: Primers utilised for the generation of the genes to express GH-GPI, 1B1-GPI and 1C1-GPI, by PCR.

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